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Inhaled ^{99m}Tc -Sestamibi clearance from Lungs and its relation to the expression of P-glycoprotein and Multidrug Resistance Protein-1

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Inhaled ^{99m}Tc -Sestamibi clearance
from Lungs and its relation to the
expression of P-glycoprotein and
Multidrug Resistance Protein-1

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Dedication

I would like to start by thanking the almighty for nurturing and guiding me through and providing me a blessed life.

My supervisors

This is a piece of work which I undertook while working as a busy NHS consultant. This meant I had very minimal time dedicated for the project. I am ever grateful to my supervisors, Prof Peters and Dr Ballinger who have been extremely helpful not only with imparting their extensive experience and knowledge but personally supporting me (enduring a lot of hassle in the process) and ensuring I stayed focussed and the project progressed smoothly.

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My family

My parents for their endless blessings, my loving wife and our children who have been a constant source of encouragement and without whose smiling faces this project would not have been possible.

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1. Abstract

P-glycoprotein (P-gp) and Multidrug Resistance Protein (MRP1) are the key cellular drug efflux transporters in a range of tissues and the radiopharmaceutical ^{99m}Tc -sestamibi has been demonstrated to be a substrate for both transporters in a number of *in vivo* and *in vitro* studies. The elimination rate of ^{99m}Tc -sestamibi from the lungs following inhalation as an aerosol has been shown to be delayed in healthy smokers versus non-smokers. It was hypothesized that this was the result of smoke-induced up-regulation of P-gp.

The aims of this study were:

To examine the relationship between the lung elimination rate of inhaled sestamibi and immuno-histochemical expression of broncho-pulmonary MRP1 and P-gp. To study the repeatability of the technique and assess the effect of various demographic factors affecting the clearance of inhaled sestamibi.

Methods:

Thirty-five participants were included in the study. They all underwent an inhaled ^{99m}Tc -sestamibi clearance study. Of these, 13 were patients undergoing surgery for primary lung cancer (5/13) or spontaneous pneumothorax (8/13). There were 22 healthy volunteers. All participants gave informed consent prior to the

study. Semi-quantitative assessment (grade 0-3) of MRP1 and P-gp expression in lung on immuno-histochemical examination was correlated with the elimination rate of sestamibi from the lung tissue.

The effect of various factors on inhaled sestamibi clearance including smoking status, age, gender and locality of residence was evaluated. The study was repeated in 10 participants to assess the repeatability and inter-observer reproducibility of the technique.

Results:

MRP1 expression was seen in 12/13 patients while P-gp in only 2/13. The mean sestamibi elimination rate was faster in patients expressing low levels of MRP1 expression (grade 0-1), mean $T_{1/2}$ of 105 ± 20 min, compared with those with higher levels of MRP1 expression (grade 2-3), mean $T_{1/2}$ of 149 ± 28 min ($P = 0.008$).

Inhaled sestamibi clearance was significantly delayed in smokers ($n=17$), mean $T_{1/2} = 142 \pm 29$ min, compared to non smokers ($n=18$), mean $T_{1/2} = 91 \pm 14$ min ($P < 0.0001$). The clearance of inhaled sestamibi was also significantly delayed in non smoking older patients >30 y ($n= 6$), mean $T_{1/2} = 101 \pm 15$ min, compared to younger patients <30 y ($n=12$), mean $T_{1/2} = 86 \pm 11$ min ($P < 0.05$), and in non smoking men ($n=7$), $T_{1/2} = 130 \pm 37$ min, compared to

women (n=11), $T_{1/2} = 105 \pm 28$ min ($P < 0.05$).

There was however no significant difference noted between urban dwellers from London (n=22), mean $T_{1/2} = 89 \pm 11$ min, compared to semi-rural dwellers (n=13), mean $T_{1/2} = 96 \pm 20$ min ($P > 0.05$).

Bland-Altman analysis revealed excellent agreement between test and re-test values with a (bias) of 4.8 min and a standard deviation of the difference (precision) of 5.7 min. There was an excellent inter-observer reproducibility seen with a Spearman Rho value of 0.96 and $p < 0.05$

Conclusion:

The study results indicate a significant correlation between the predominant transporter in lung (broncho-alveolar epithelium) MRP1 and inhaled ^{99m}Tc - Sestamibi clearance. Inhaled ^{99m}Tc - Sestamibi clearance is a safe and reproducible technique.

Smoking, increasing age and male gender appear to significantly prolong the lung clearance of inhaled sestamibi. There was however no definite effect of environmental status on the clearance rate observed.

2. Introduction

Chemotherapy in lung cancer was first studied in the late 1960s and since then has been used extensively in this disease (1,2).

The use of chemotherapy has resulted in a fivefold increase in median survival and a long-term disease-free survival of over 3 years in 5% to 10% of small cell lung cancer (SCLC) patients and 4-15% improvement in median 5 year survival in non small cell lung cancer (3).

Despite advances in cancer treatment, multidrug resistance (MDR) remains a major clinical problem resulting in poor patient outcome due to limited therapeutic options. This remains a major issue in a number of cancers, including leukaemia and carcinomas of the prostate, breast, lung and ovary. Significant efforts to gain a better understanding of the mechanisms that result in the development of drug resistance have been undertaken in order to improve the outcomes for current chemotherapy regimens in cancer treatment. MDR can develop in response to a specific drug or drug combination, due to phenotypes that typically confer resistance to a variety of agents.

The mechanisms of resistance development are often diverse, including **(a)** insufficient uptake of water-soluble drugs by specific transporters, **(b)** alterations in cellular pathways which result in a

reduced capacity of cytotoxic drugs to kill cells, or **(c)** increased drug efflux from cancer cells.

One of the important mechanisms of development of drug resistance has most often been linked to the over expression of Permeability glycoprotein (P-gp), a 170-kD ATP dependent membrane transporter that acts as a drug efflux pump. (4,5)

3. Multidrug Resistance and the ABC transporters

P-gp (permeability glycoprotein)

The term “multidrug resistance” (MDR) describes the phenomenon where tumor cells develop a broad cross-resistance to a variety of structurally and functionally different cytotoxic drugs (6). It was first described in certain tumor cells that are protected against anticancer agents as a result of P-glycoprotein over expression (7).

Preclinical studies have shown that resistance of tumour cells to cytostatic drugs derived from naturally occurring toxins is often closely related to the presence of a unique 170 kDa glycoprotein, called P-glycoprotein (P-gp) which is located on or in the cell membrane of the tumor cells. (7-9). This molecule acts by expelling these cytostatic drugs from the cytoplasm, causing drug

resistance, at least *in vitro* (10-13). Studies have demonstrated expression P-gp in normal human tissue including the lungs. (14-16). P-gp expression has also been demonstrated on (and in) cells with secretory and excretory functions (proximal tubules, bile canaliculi, glands in the prostate and breast, sweat glands, intestinal epithelia), which suggests a physiological role in removal of potentially toxic substances (17-19).

A significant understanding of the functional role of P-glycoprotein in drug transport was described by Schinkel et al using P-glycoprotein-deficient mice (20, 21). They elegantly demonstrated the result of inhibition of the mouse P-glycoprotein gene leads to a deficiency in the blood-brain barrier and increased sensitivity to drugs. Also, they compared the drug concentrations in various tissues in P-glycoprotein-expressing and P-glycoprotein-deficient animals and demonstrated the cell protective value of this transporter *in vivo* (22).

P-gp Structure and interaction:

P-gp, encoded by the human MDR1 gene located on chromosome 7, is one of 48 known human ATP binding cassette (ABC) transporters. It is a 170 kDa molecular weight phosphoglycoprotein consisting of 1280 amino acids and consisting of two ATP binding cassettes and two transmembrane regions. (23). The N-terminal half of the molecule contains 6 transmembrane domains, followed by a large cytoplasmic domain with an ATP-binding site, and then a second section with 6-transmembrane domains and an ATP-binding site that shows over 65% of amino acid similarity with the first half of the polypeptide (24).

P-gp can recognise and bind a large variety of hydrophobic natural-product drugs as they enter the plasma membrane. These include many of the commonly used anticancer drugs, such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol, as well as many commonly used pharmaceuticals ranging from antiarrhythmics and antihistamines to cholesterol-lowering statins (25) and HIV protease inhibitors (26). Binding of these drugs results in activation of one of the ATP-binding domains, and the hydrolysis of ATP causes a major change in the shape of P-gp, which results in release of the drug into the extracellular space

(27). Hydrolysis of a second molecule of ATP is needed to restore the transporter to its original state so that it can repeat the cycle of drug binding and release (28, 29). Although the detailed mechanism of action of other ABC transporters is not known, it is presumed that the ATP binding cassette acts as the engine for the transport mediated by members of this large family of transporters.

The discovery of P-gp and the demonstration of its widespread expression in many human cancers was a significant step towards understanding the development of drug resistance. However, it was found that many multidrug-resistant cancers, such as lung cancers, rarely express P-gp (30-33). P-g-p expression was at best heterogeneous, ranging from rare scattered cells to a positive pattern for nearly all cells considered, without any relationship with pathologic and clinical prognostic variables (31).

As a result, a search for other efflux pumps that may be responsible for development of MDR was initiated.

Multidrug resistance associated protein 1 - MRP1

Using a multidrug-resistant lung cancer cell line as a model system, Deeley, Cole and colleagues (34) cloned another ABC family member, known as MRP1 (multidrug resistance associated protein 1), a 190 kDa molecular weight protein. It was originally identified as being responsible for MDR in a doxorubicin-selected human small cell lung carcinoma cell line. Even though these cells displayed an MDR phenotype similar to that previously associated with P-gp over expression, resistance was found to be P-gp-independent (34, 38). MRP1 is also widely expressed in many human tissues and cancers (35).

MRP1 has since been closely linked to the development of clinical MDR in several types of cancer (36, 37), and an extraordinarily wide variety of transport substrates have been identified, showing that it has a broad spectrum of anticancer drug transport activity (34).

MRP1 structure and interaction

MRP1, unlike MDR1 proteins, transports negatively charged natural products and drugs that have been modified by glutathione (GSH), conjugation, glycosylation, sulfation, and glucuronylation.

In some cases co-transport of glutathione with positively charged drugs, such as vinblastine, can occur. MRP1 also transports diverse physiological substrates such as folates, GSH and GSH disulfide (GSSG), as well as sulphate-, GSH- and glucuronide-conjugates of steroids, leukotrienes and prostaglandins (37, 39). The activities of MRP1 are important in normal cellular processes, such as export of endogenous intermediates and protection from toxic insult. MRP1 over expression in cancer cells allows the elimination of a wide range of therapeutic agents, and due to its ability to influence cellular GSH-GSSG balance through export of GSH and its derivatives, MRP1, could play an important role in the cellular response to oxidative stress induced by drugs or associated with various disease states.

Up-regulation of MRP1 has been observed in a variety of solid tumours such as those of the lung, breast and prostate, and this transporter has been clearly implicated as having a role in the clinical drug resistance behaviour of several of these cancers.

The most common type of lung cancer, non-small cell lung carcinoma (NSCLC), is typically chemoresistant and MRP1 was found to be over expressed in a large proportion of these cancers prior to treatment exposure. MRP1 expression correlated strongly

with poor response to chemotherapy and poor overall survival (38).

MRP1 expression was also predictive of poor response to chemotherapy in small cell lung carcinoma (SCLC) (38).

MRP1 and P-gp expression were noted to be increased in SCLC metastases detected at relapse, suggesting a role for these transporters in cell survival during metastasis and chemotherapy (40).

Prime drug efflux transporter in lungs; P-gp Vs MRP1

While studies have revealed high levels of P-gp expression in most tumours derived from tissues expressing P-gp, a discrepancy was sometimes found between the level expressed in the tumour and adjacent normal tissues, particularly within the lungs (13-15).

Wright et al and Berger et al (41, 42) interestingly observed strong expression of MRP1 in normal broncho-alveolar epithelium with a distinctly increased expression in tumour cells (adenocarcinomas, carcinoid tumours and SCLC tumours) as compared with the normal cell counterpart. They however observed a very weak or negative expression of P-gp and limited expression of lung resistance protein (LRP) both in the normal lung tissue and chemotherapy-naïve tumour tissue. Berger et al (42) observed that

P-gp expression in lung tumours was only increased in those patients who had been exposed to chemotherapy prior to surgery.

Despite the positive results of a small, randomized study of the addition of verapamil (as a P-gp inhibitor) to chemotherapy in untreated NSCLC (40), three large randomized trials failed to show any benefit of agents that reverse the MDR phenotype in terms of the response to chemotherapy or the length of survival. One of these trials was performed in untreated patients with SCLC and failed to demonstrate any benefit of adding verapamil to chemotherapy (43).

Narasaki et al. investigated the expression of MRP mRNA and drug sensitivity in lung cancer cell lines and tested the modulating effects of verapamil. They demonstrated clear correlations between the MRP gene level and the sensitivity to etoposide and doxorubicin usage. They also demonstrated various levels of MRP gene expression but not the MDR1 gene in the cell lines and also noted that the levels were higher in NSCLC than in SCLC cell lines (44).

MRP1 appears to be expressed at a basal level in normal lung tissue and it is therefore important to measure MRP1 expression in normal cells as a baseline and compare to that in malignant cells (45).

Other ABC transporters

Other ABC transporters have also been implicated in the development of drug resistance and are of potential significance, including the *MDR2* gene product (46, 47), a protein named SPGP (sister of P-gp) (48), and ABC A2 (49). Other members of the ABC transporter family in addition to the MDR, MRP, and MXR family members have also been implicated in clinical cancer drug resistance or in drug transport in humans and efforts to explore their patterns of expression and determine their function are ongoing.

4. ^{99m}Tc-Sestamibi (methoxyisobutyl isonitrile) for demonstrating P-gp / MRP1 function

The technetium-99m-labeled complexes, sestamibi (^{99m}Tc]-MIBI) and tetrofosmin (^{99m}Tc]-TFS), originally designed for myocardial perfusion studies, are recognized by P-gp and MRP1 as transport substrates.(50–53). These agents are cationic and modestly hydrophobic, thus sharing common characteristics of cytotoxics comprising the MDR phenotype. (54, 55)

Many *in vitro* and *in vivo* studies have demonstrated correlation between the uptake and/or retention of these radiotracers and the expression levels of P-gp or MRP1 in tumour cells (50, 52, 56–61). Therefore, [^{99m}Tc]-MIBI and [^{99m}Tc]-TFS have been proposed as imaging probes for MDR assessment and to monitor the efficacy of MDR modulators. They have also been used as imaging agents in the evaluation of breast, bone, thyroid, parathyroid and lung tumours (62-65).

5. Effect of smoking on P-gp and MRP expression and functionality

Little is known about the factors affecting functionality of P-gp or MRP1 in the lungs. *Ex vivo* data from human lung suggests that pulmonary P-gp is up-regulated by cigarette smoke (66, 67) and MRP1 is down-regulated in chronic obstructive pulmonary disease (COPD) (68). Van der deen et al demonstrated that exposure to cigarette smoke extract (CSE) up regulates MRP1 expression and also competitively inhibits MRP1 activity in the bronchial epithelium, resulting in increased CSE related cytotoxicity. It was therefore suggested that MRP1 had a possible protective role against COPD (69).

Sam et al demonstrated in smokers, tobacco chewers and alcoholics, an increased polymorphism of the ABCB1 gene that encodes P-gp, which was associated with increased risk of aerodigestive tract cancers (70).

In Volm et al. study, 58% of lung cancers in smokers, compared with 9% in non-smokers, expressed P-gp (71). In the study by Lechapt-Zalcman et al (15), however, different levels of expression of P-gp in bronchial tissue had been observed between individuals in the two groups (smokers vs non smokers) evaluated, with a

higher mean in the smokers group, although with no statistically significant difference. This raises the question as to whether smoking does result in increased P-gp expression or whether there is increased functionality of P-gp in these patients.

6. Inhaled ^{99m}Tc -sestamibi clearance imaging in human lungs

Recently, Ruparelia et al (72) described the technique of studying the clearance of inhaled aerosolised ^{99m}Tc -sestamibi from human lungs in healthy volunteers. This involved the participants inhaling aerosolised ^{99m}Tc -sestamibi via a ventilation kit during relaxed tidal breathing in the sitting position. Regions of interest were placed around the periphery of both lungs, excluding the central airways. Time–activity curves were generated for each lung, summed and corrected for the physical decay of ^{99m}Tc .

They observed that the lung elimination rate of ^{99m}Tc -sestamibi following inhalation as an aerosol in normal non-smoking humans is much slower than would be expected from its molecular size and physical properties and about half as fast as that of the hydrophilic, radio-aerosol, technetium-99m-diethylenetriaminepentaacetic acid (^{99m}Tc -DTPA) (72). No activity was seen in the airways or GI tract

for several hours after ^{99m}Tc -sestamibi inhalation, confirming that elimination of radioactivity took place across the gas-blood barrier. Unlike inhaled ^{99m}Tc -DTPA, the lung elimination rate of which is markedly accelerated in cigarette smokers (73), ^{99m}Tc -sestamibi elimination rate was noted to be markedly *delayed* in smokers compared with non-smokers. Also in contrast to ^{99m}Tc -DTPA, ^{99m}Tc -sestamibi elimination was not accelerated in interstitial lung disease. It was, however, significantly delayed in ex-smoking patients with chronic obstructive pulmonary disease (73).

The lung elimination rate of the related compound, ^{99m}Tc -tetrofosmin, was also slower than that of ^{99m}Tc -DTPA, but in contrast to ^{99m}Tc -sestamibi interestingly not delayed in cigarette smokers (74).

While ^{99m}Tc -DTPA crosses the gas-blood barrier in the lungs by passive diffusion through inter-epithelial junction gaps, a lung elimination rate of inhaled ^{99m}Tc -sestamibi slower than that of ^{99m}Tc -DTPA was therefore hypothesized to be due to intracellular ^{99m}Tc -sestamibi binding in airway epithelium (74). It was also suggested that the elimination would be further delayed if lung P-gp pumped the tracer back into the airways and that the slow elimination rate seen in smokers was the result of smoke-induced

upregulation of P-gp. A similar smoke-induced delay of ^{99m}Tc -terofosmin clearance was not seen because this compound is less P-gp avid (72).

7. Current techniques of demonstrating P-gp

expression in cells:

To date the gold standard for demonstrating the presence of P-gp in cells has been histopathological demonstration of the presence of P-gp in the tissue specimen. Various techniques including immunohistochemical, immunocytochemical (14) and Western blot analysis have been performed on histological specimens to demonstrate the level of expression of P-gp in the cells.

Several different monoclonal antibodies have been used for the immunohistochemical quantification of P-gp expression, some of which bind extracellular epitopes and some intracellular epitopes.

However, immunohistochemical assessment of tissue P-gp expression is not without its share of problems. Tissue P-gp expression is antibody- as well as tissue-dependent and described as heterogeneous throughout tissues (31). Immunoreactivity is not

specific which could result in false positive results (12). Other potential problems include sampling errors, tissue fixation effects (14) or failure of P-gp expression to translate to function.

Apart from histological demonstration of P-gp in cells, intravenous injection of ^{99m}Tc -sestamibi is the current technique used to look at the expression of P-gp in vivo and predict chemotherapy resistance in patients. Although studies have shown this to be a reasonably accurate technique (75- 77), there is a significant overlap noted between responders and non-responders in these studies affecting the specificity of the technique. There are no prospective studies however performed to confirm the value of the technique.

Whilst immunohistochemistry is regarded as the gold standard for P-gp expression, retention and elimination of a P-gp substrate represents an attractive alternative for the *in vivo* measurement of functional expression and moreover is independent of sampling error.

8. Aims and Rationale of the study

The primary aim of this project was to explore the relation between broncho-pulmonary P-gp / MRP1 expression and the transfer of inhaled ^{99m}Tc -sestamibi from airspace to blood. The secondary aims were to determine the repeatability of inhaled ^{99m}Tc -sestamibi clearance technique and explore the effect of patient and demographic factors on the clearance of inhaled ^{99m}Tc -sestamibi. The primary aim was addressed firstly by correlating the inhaled ^{99m}Tc -sestamibi elimination rate from the lungs with immunohistochemical expression of P-gp and MRP1 in surgically resected normal lung tissue.

We would also look at the location of these transporters within the broncho alveolar epithelium on immunostaining which would support the hypothesis of these transporters playing a role in the clearance of inhaled ^{99m}Tc -sestamibi from the lungs.

The repeatability of the technique was assessed in 10 patients who underwent a repeat study within 2-4 weeks of the primary study. An inter-observer reproducibility was assessed in 5 patients. The effect of patient factors including smoking status, age, gender and the possible effect of locality of patient residence

was correlated with inhaled ^{99m}Tc -sestamibi clearance in these patients.

If the presumed hypotheses were proven, inhaled ^{99m}Tc -sestamibi clearance may prove to be a very useful technique to assess the functional expression of P-gp / MRP1 in patient lungs and obviate the necessity to obtain tissue samples by invasive biopsy. It would provide a simple, reproducible, economical outpatient procedure with minimal exposure to radiation (~0.5 mSv compared to 8 mSv for iv ^{99m}Tc -sestamibi study) providing crucial information regarding functional status of lung drug efflux transporters in patients.

This crucial information may help tailor the treatment to the patient and may also help understand the role these transporters play in the development of lung pathology.

9. Study design, Materials & Methods

Institutional Ethics committee approval was received for this prospective study. Informed written consent was obtained from all participants in the study. The study was conducted in compliance with the principles of the Declaration of Helsinki (1996), the principles of GCP and all of the applicable regulatory requirements.

Patient characteristics

35 participants were included in the study. 13 of these participants were patients who were planned to undergo lung surgery recruited from the thoracic surgical clinic dealing with the management of patients with lung cancer and spontaneous pneumothorax. 22 were normal healthy volunteers recruited via the trial recruitment service at King's College London.

Inclusion criteria:

We included patients aged 18 years or more. They were all able to provide informed consent and able to lie supine for 40 minutes under the gamma camera.

Exclusion criteria:

We excluded patients who were less than 18 years of age, pregnant patients or if they were taking known P-gp / MRP1 modulating medication.

Nineteen potential patients from the thoracic surgical clinic who were planned to undergo surgery for primary lung cancer or recurrent pneumothorax were screened. Thirteen patients undergoing surgery for primary lung cancer (5/13) or spontaneous pneumothorax (8/13) consented to the study.

A further 64 healthy volunteers were screened of whom 22 agreed to participate in the study.

All the participants underwent an inhaled sestamibi clearance study. The clearance study was repeated in 10 participants to confirm the repeatability of the technique.

Of the 35 participants, there were 17 who were smokers and 18 non smokers. 17 were male and 18 female while 13 participants resided in a semi rural location (West Sussex) and 22 resided at an urban (London) address.

Age of the participants ranged between 20 -78 with a mean of 38 years. Concomitant medication intake was recorded in all the participants. None of the participants were noted to be on P-gp or MRP1 modulating medications.

Inhaled ^{99m}Tc-sestamibi clearance study acquisition

parameters

400 MBq of ^{99m}Tc-sestamibi was placed in the Smart Vent nebuliser (Diagnostic Imaging Ltd). The nebulised radioactive tracer was inhaled for 1- 5 minutes by the participant, sitting upright, via a mouth piece until a count rate of at least >0.3 Kcounts per second was recorded. This was in keeping with the departmental guideline (ventilation / perfusion scintigraphy) to ensure diagnostic quality images were obtained in all patients. Participants were instructed to lie supine on couch of the Siemens Symbia TM dual head large field of view gamma camera. Anterior and posterior images of the chest were acquired dynamically at a rate of 2 seconds per frame starting immediately after inhalation was completed. Matrix size was 64 X 64 and no zoom applied. The imaging was carried on for 40 minutes.

Images were transferred to Hermes (Hermes Medical, Stockholm) workstation for analysis.

Radiopharmaceutical purity was tested on every batch used in the patients. All batches demonstrated radiochemical purity typically >97% as confirmed by daily quality control procedures.

This was to ensure any impurities like colloids which could be taken up by the alveolar macrophages and potentially affect the

study were excluded. All the images were evaluated for the presence of free pertechnetate in the radiopharmaceutical by including the thyroid and the stomach in the field of view. None of the studies demonstrated contamination with free pertechnetate.

Analysis of Inhaled ^{99m}Tc-sestamibi clearance

Analysis of the dynamic images was performed on Hermes work station using the default dynamic study analysis software package. Regions of interest were drawn around both lungs on the anterior and posterior images. Time activity curves were generated for both lungs and the effective half life ($T_{1/2}$) for clearance of activity from the lungs over a period of time was calculated by fitting curves to first order equation. The individual lung clearances were obtained as geometric mean of the anterior and posterior clearance results. In 10 participants the inhaled ^{99m}Tc-sestamibi clearance study was repeated within 2-4 weeks of the initial study, to assess the repeatability of the technique. In 5 participants the inhaled sestamibi clearance was assessed by a second experienced nuclear medicine clinician. The individual clearance for each lung was compared between the two readers to assess the inter-observer reproducibility.

A first order exponential curve fitting was used to assess the elimination rate as we also wanted to assess the effect of varying breathing techniques in different participants.

Tissue Immunohistochemistry:

Paraffin embedded lung tissue obtained from the participants who underwent surgery for underlying disease was subjected to immunohistochemical examination for the presence of P-gp and MRP1. Immunohistochemistry was performed using the Ventana BenchMark ULTRA staining machine (Ventana Medical Systems Inc, Tucson, AZ, USA). Immunostaining procedure was standardised using human Gut epithelium as control tissue.

(Figures 1a and 1b) JSB-1 mouse antibody (AbCam ab 3366, AbCam, Cambridge, UK)) was used for P-gp detection (1/50) and Anti-MRP-1 mouse antibody MRPm5 (AbCam ab 24102, AbCam, Cambridge, UK) was used for MRP-1 detection (1/100).

Antigen retrieval was performed following incubation of in Ultra CC1 (Tris/Borate/EDTA buffer pH 8-8.5), for 36 min, at 95°C.

Antibody was diluted in Antibody Diluent (Dako Real S2022, DAKO, Denmark).

Primary Antibody was incubated at 36°C for 32 min and secondary antibody was incubated at 36°C for 30 min.

Tissue was counterstained with hematoxylin II for 4 min followed by Bluing Reagent for a further 4 min. Following this, slides were washed in soapy water, dehydrated through serial alcohol dilutions - 70% and 100% twice for 2-3 min each and twice with Xylene for 3 min each. The slides were mounted in Eukitt (Sigma 03989, Sigma-Aldrich, Dorset, England) for analysis.

A semi quantitative method for evaluating P-gp and MRP-1 expression was based on the strength of immunostaining, which was graded into 4 categories;

Negative = 0, weak =1, moderate = 2 and strong = 3. (fig 1c)

The immunohistochemical analysis of P-gp and MRP1 expression in the normal lung tissue surrounding the cancer / bullous lung tissue and within the cancerous lung was performed by an experienced lung histopathologist.

10. Statistical analysis

Statistical advice was sought from a statistics expert.

The study was powered to demonstrate a difference of 20% or more in the mean inhaled sestamibi clearance between patients expressing negative / weak drug transporter status (Grades 0-1) compared to moderate / strong drug transporter status (Grades 2-3) as assessed by immunohistochemistry and a minimum of 13 patients was advised.

Repeatability of the Inhaled ^{99m}Tc -sestamibi clearance technique was assessed using Bland-Altman analysis to test agreement between the test and retest values. A Spearman Rho calculator was used to test the inter-observer reproducibility of the technique. Clearance time for 50% of inhaled ^{99m}Tc -sestamibi from the lungs ($T_{1/2}$) was compared between different groups of patients to study the effect of: smoking, gender, age and the quality of inhaled air as assessed by the dwelling status of the patient, urban or rural (as an expected surrogate).

Mean inhaled sestamibi clearance values and the standard deviation were calculated.

Parametric testing using *Student's t-test* (2 tailed) and non-parametric testing using *Mann-Whitney test* and *Spearman's Rho* test were applied to the data.

11. Results

The individual lung clearances were obtained and compared with each other. A Spearman Rho test was used to check the correlation between the right and left lung clearances (Table 1).

This confirmed an excellent correlation between the individual lung clearances, $p < 0.05$ with an R value of 0.95. We obtained the mean of the two lung clearance values as a single inhaled ^{99m}Tc -sestamibi clearance for each patient.

a) Immunohistochemistry and inhaled ^{99m}Tc -sestamibi clearance results (n=13) 4 non-smokers and 9 smokers

Expression of P-gp was negative, grading 0, in 11 / 13 (Fig 2a) and only weakly positive, grading 1, in 2/13 patients.

MRP1 expression was demonstrated in 12/13 patients (Fig 2b) with negative MRP1 expression noted in only 1 patient within the healthy broncho-alveolar epithelium.

The MRP1 staining was noted to be located predominantly in the apical aspect of the broncho-alveolar lining epithelium with some staining also seen at the basolateral aspect. (Fig 2c)

Clearance of inhaled ^{99m}Tc -sestamibi was significantly delayed in patients demonstrating upregulated MRP1 levels, grading ≥ 2 , $n=7$

($T_{1/2} = 148.8 \pm 27.8$ min) compared to those expressing low MRP1 levels, grading ≤ 1 , $n=6$ ($T_{1/2} = 104.8 \pm 20.1$ min) $p=0.008$ (Fig 3a and 3b).

Non-smokers ($n=4$) demonstrated low levels of MRP1 expression 0 ($n=1$) and 1 ($n=3$) while MRP1 levels were up regulated in 9 smokers, 1 ($n=1$), 2 ($n=5$) and 3 ($n=3$).

In contrast, there was no difference in the P-gp expression between smokers and non-smokers.

In all 5 patients with lung cancer, MRP1 levels were upregulated in the cancer tissue (grade 2-3) compared to the normal epithelium (grade 1-2).

b) Reproducibility (Fig 4a and 4b)

Ten participants underwent repeat inhaled ^{99m}Tc -sestamibi clearance study within 4 weeks of the initial study.

Bland-Altman analysis of the data revealed that in 9 / 10 (Fig 4a) participants the difference between the test and retest values was within two standard deviations with a (bias) of 4.8 min and a standard deviation of the difference (precision) of 5.7 min suggesting excellent agreement. In one patient in whom the

difference was $>2SD$ it was noted that the participant who was a smoker, had stopped smoking at least 2 weeks prior to the second test which may explain the significant difference between the two results. The results including the 10th patient are presented in Fig 4b with a (bias) of 7.1 min and a standard deviation of the difference (precision) of 9.3 min.

There was an excellent inter-observer reproducibility seen with a Spearman Rho value of 0.96 and $p < 0.05$ (Table 2a) for the individual lung clearances. The Spearman Rho was 0.99 and $p < 0.001$ (Table 2b) between the 2 reviewers for the clearance values measured as a mean of both lung clearance values for each patient.

c) Smokers (n = 17) vs Non Smokers (n = 18) (Fig 5)

Mean inhaled MIBI clearance $T_{1/2}$ for smokers was 142.2 ± 29.3 min and that for non-smokers was 90.5 ± 14.1 min demonstrating a significant ($p < 0.05$) delay in inhaled ^{99m}Tc -sestamibi clearance in smokers.

As smoking seemed to have a significant impact on the inhaled sestamibi clearance, we studied the effect of gender, age and environmental status in non-smokers separately to eliminate any confounding bias.

d) Gender - Male (n = 7) vs Female n = 11) non smokers (Fig 6)

The calculated mean T1/2 values for males was 130.3 ± 37.0 min which was delayed when compared to female participants with a mean T1/2 value of 105.6 ± 27.9 min ($p = 0.037$).

In the smokers, there was no significant difference ($p=0.211$) seen between the male participants ($n=10$) 151.3 ± 28.9 min and females ($n=7$), 129.6 ± 29.1 min.

e) Rural Vs Urban (n = 13 vs 22) (Non smokers London n = 12 vs semi rural n = 6) (Fig 7)

There was no significant difference in the mean T1/2 times between non-smoking participants living in an urban area (London; 89.0 ± 11.02 min ($n = 12$) compared to participants living in a semi rural area (West Sussex; $95.8 \text{ min} \pm 20.0 \text{ min}$. $p = 0.36$)

In the smokers, there was no significant difference ($p=0.87$) seen between the London participants ($n=7$) 140.8 ± 35.3 min and west sussex participants ($n=10$), 143.3 ± 26.3 min.

f) Age (Non smokers <30 years n = 12 vs >30 n = 6) (Fig 8)

There was a trend of increased inhaled ^{99m}Tc -sestamibi clearance times with age. There was faster clearance seen in those <30

years, $n=12$, exhibiting a mean clearance of 86.0 ± 11 min compared to those >30 , $n=6$ a mean clearance of 100.6 ± 14.5 min ($p = 0.027$).

In the smokers, there was no significant difference ($p=0.97$) seen between the participants <30 years 142 ± 24.6 min ($n=8$) and those >30 years age ($n=9$), 142.4 ± 34.5 min.

12. Discussion:

The prominent expression of P-gp and MRP1 in the human lung (78) suggests that these transporters may play an important role in the protection against endogenous or exogenous toxic compounds entering the lung. The delivery of pulmonary drugs to reach their site of action may also be affected by the presence and activity of these drug transporters (79).

It is known that in drug-resistant cells both P-gp and MRP1 may be over expressed at the same time (80, 81).

However there remains a debate about the role of P-gp as a major drug transporter in human lungs.

Inhaled ^{99m}Tc Sestamibi clearance and drug transporter expression in lungs

In the present study very little or no P-gp expression was identified in the broncho-alveolar epithelium while there was clear MRP1 expression noted on immunohistochemical analysis of normal lung tissue and lung tumours. Furthermore, we demonstrate a significant delay in the clearance of inhaled sestamibi, an established MRP1 substrate, from the lungs in patients with higher MRP1 expression (Grade 2 & 3) compared to those with low or no MRP1 expression (Grade 0 & 1) (Fig 4a and 4b).

The expression of P-gp within the broncho-alveolar epithelium and its role in facilitating transfer of the substrates has been researched previously. The current opinion is that P-gp is mainly expressed in the larger tracheo-bronchial airway epithelium while there is no significant expression in broncho-alveolar epithelium (82).

Wright et al and Berger et al, in their studies (41,42), interestingly observed strong expression of MRP1 in normal broncho-alveolar epithelium with a distinctly increased expression in tumour cells as

compared with the normal cell counterpart, while they observed very weak or negative expression of P-gp and limited expression of lung resistance protein (LRP) both in the normal lung tissue and chemotherapy naïve tumour tissue. Berger et al (42) also observed that P-gp was expressed significantly higher ($p < 0.05$) in tumor tissues for patients who had undergone neo-adjuvant chemotherapy before surgery.

Hendrikse et al (83) demonstrated functional modulation of these proteins with specific blockers while Schinkel et al (84) observed in the lung tissue of the P-gp knockout (-/-) mice that the level of ^3H -digoxin (P-gp substrate) was low compared to other organs suggesting a limited role for P-gp as a drug transporter in the lungs. In keeping with the literature evidence the current study did not identify significant P-gp expression in the chemotherapy naïve lung tissue.

To support the transporter effects of these proteins, their localisation is a key factor. The likely location of MRP1 and other ABC transporters within the broncho alveolar epithelium has been described variably in the literature. This has been well summarised and best explained by van der deen et al (82) in their study.

In this study we were able to clearly demonstrate the cytoplasmic presence of MRP1 and its preferential apical expression in the bronchoalveolar epithelium (fig 2c). This is a finding which has not been well documented in the literature previously.

The expression of MRP1 was observed in basolateral aspect of the ciliated epithelial cells (Fig 2c).

We speculate that while the transfer of the inhaled ^{99m}Tc -sestamibi is driven by the difference in the action potential across the cell membrane, once it is inside the cell (cytoplasm) the apical MRP1 expression likely mediates its transfer across the membrane back into the airspace. The basolateral expression of the MRP1 within the cells we feel would likely facilitate the transfer of the intracellular ^{99m}Tc -sestamibi into the capillaries (circulation) as noted by the appearance of the tracer in the systemic circulation during the later part of the study (fig 9).

Decreased or increased functional MRP1 expression may have an impact on development and/or progression of lung diseases and protection against air pollution and inhaled toxic substances.

Indeed, Van der deen et al observed lower MRP1 expression in patients with chronic airway disease and postulated that the lower

expression of MRP1 or the down-regulation of MRP1 may result in an increased vulnerability to, and subsequent development of, chronic airway disease (68).

Imaging the kinetics of known drug transporter substrates across the alveolo-capillary membrane using inhaled clearance technique provides a very interesting model to directly interrogate the effects of drug transporters on a large variety of inhaled pulmonary therapeutic agents, such as corticosteroids and sympathomimetics that may be substrates for these transporters in the lung or modulate their activity. Functional inhalation studies would also provide an excellent opportunity to demonstrate effects of inhaled toxic substances, as postulated by van der deen et al (68).

Inhaled ^{99m}Tc -sestamibi clearance is a functional imaging technique that was first described by Ruparelia et al in 2008 (72). They studied the clearance of inhaled sestamibi in normal human volunteers and patients with chronic obstructive pulmonary disease (COPD). They observed significant differences in inhaled sestamibi clearance rates between smokers and non-smokers while no significant difference was seen between healthy volunteers compared to COPD patients. They postulated that the

delayed clearance of sestamibi across the alveolo-capillary barrier was regulated by active binding and efflux of sestamibi by P-gp expressed in the lung tissue (72). They also postulated that delayed sestamibi clearance in smokers compared to non-smokers was due to active efflux of inhaled sestamibi mediated by increased P-gp expression in the alveolar epithelium, pumping it back into the airspaces (74). Interestingly, this group showed no difference between smokers and non-smokers with respect to the lung clearance rate of inhaled tetrofosmin (74). There was however no immuno-histochemical correlation available to confirm the postulation.

The findings of van der deen (68) and Ruparelia (72) are interesting particularly in the COPD population. While van der deen et al observed low MRP1 expression in COPD subjects, Ruparelia et al demonstrated faster clearance of inhaled sestamibi in these subjects in their study.

The initial response to cigarette smoke exposure is one of increased MRP1 expression as a protective effect. However with prolonged heavy smoking, there is a competitive inhibition of the MRP1 thereby leading to loss of protective effect and development of COPD which would explain the findings of both van der deen et al and Ruparelia et al.

While immunohistochemistry is regarded as the gold standard for determination of MRP1 expression in lung epithelium, the technique is limited by heterogeneous expression of drug transporters in the lung tissue. The demonstration of expression is dependent on antibody selection and also remains tissue-dependent (31). Immunoreactivity is not specific which could result in false positive results (11). Other potential problems include sampling errors, tissue fixation effects (14) and failure of protein expression to translate to function.

In contrast, functional imaging using radiolabelled drug transporter substrates represents an attractive alternative approach for the *in vivo* measurement of functional expression and moreover is independent of histological sampling errors.

On review of the literature, functional imaging studies demonstrating the role of MRP1 in lungs remains very limited. Apart from a single animal study by Okamura et al (85) who demonstrated MRP1 as the prime drug transporter across the lung epithelium with P-gp playing no significant role, there is a lack of

functional imaging studies demonstrating the role of MRP1 in lungs.

Our study is the first *in vivo* study to our knowledge that demonstrates a significant correlation between the functional inhaled ^{99m}Tc - sestamibi clearance technique and the immunohistochemical expression of MRP1 in human lungs. We were able to demonstrate the localisation of the MRP1 within the broncho-alveolar epithelium which would support the hypothesis that the increased expression of the transporter protein (MRP1) in these cells, likely regulates the clearance of inhaled sestamibi.

Interestingly we observed while there was expression of MRP1 in the normal broncho-alveolar epithelium, it was consistently upregulated in the lung tumour tissue as seen on immunohistochemistry although the number of patients with lung cancer was small, $n=5$. This is similar to the observations of Wright et al and Berger et al (41, 42). However it is difficult to know whether the increase in MRP1 expression noted in the normal lung tissue was already increased prior to the onset of cancer or is a result of the systemic effect of the cancer.

It is likely that the increased MRP1 expression in the cancer tissue is a combination of factors affecting the MRP1 expression and the inherent protective effect developed by the cancer cell to escape the immune system response.

Factors affecting MRP1 expression and inhaled sestamibi clearance

There are very few studies assessing the factors affecting MRP1 expression in lungs while inhaled ^{99m}Tc -Sestamibi clearance is a novel technique.

Cigarette smoking appears to be one of the main factors affecting MRP1 expression in human lungs. Van der deen et al (67) observed that exposure to cigarette smoke upregulated MRP1 levels in humans. However, MRP1 expression, as assessed by immunostaining, was lower in bronchial biopsies of COPD patients as compared to that in healthy patients as well as in patients affected by severe COPD versus those with a mild to moderate form of the disease. (66) Consequently, they postulated that (69) following exposure to cigarette smoke there is upregulation of MRP1 expression in lungs as a protective mechanism. However with sustained and significant smoking exposure resulted in

competitive inhibition of the protective MRP1 up regulation and thereby resulting in cigarette smoke induced damage to the alveolar epithelium.

However in a different study, MRP1 mRNA levels were not statistically different in healthy smokers, ex-smokers and non smokers in a study by Breechot et al. (80)

These are important observations as they highlight the importance of the limited understanding we have regarding these transporters and their functional significance in the development of pathology and its progression.

Wright et al (41) and Berger et al (42) looked at the association between MRP1 expression in human lung tumours and patient characteristics including age and gender. Both studies did not find a significant association between the MRP1 score and the patient's age, gender, (41,42) tumor size, presence of metastases, stage, or grade of differentiation, (41). However, they observed that MRP1 expression was found in tumours with lower TNM stage (stage 1) compared to higher TNM stage tumours possibly suggesting a protective role for MRP1.

The results of this study reveal significant correlation between delayed ^{99m}Tc -Sestamibi clearance and smoking status, increasing age and male gender. Smoking was the single most important factor affecting clearance of inhaled sestamibi from the lungs. When we analysed the data for the effect of age, gender and locality of residence on inhaled sestamibi clearance including the smokers, there were no significant differences observed. However, upon exclusion of smokers, we observed increasing age (>30years) and male gender appear to delay inhaled sestamibi clearance ($p < 0.05$). There was however no significant correlation between locality of residence of participants and inhaled sestamibi clearance. This was analysed to assess the possible effect of atmospheric pollution in urban areas compared to those living in semi rural areas on inhaled sestamibi clearance. This however may not be unexpected and may be difficult to evaluate given the nature of travel and lifestyle of individuals today.

We also analysed the inhaled sestamibi clearance in smokers separately to see if the effect of age and gender still persisted. Interestingly we did not observe any significant impact of age or gender on inhaled sestamibi clearance between different groups of smokers. This suggested smoking was a single significantly strong

factor which affected clearance of inhaled sestamibi independent of other variables including age and gender.

We ensured none of the participants were on a P-gp or MRP1 modulating medication, including contraceptive medication, which could have biased the results.

Whilst the results are significant, they cannot be attributed directly to the expression of MRP1 alone as we have immunohistochemistry confirmation in only a small number of 13 patients. These results need to be tested in larger trials with immuno-histochemical comparison.

Reproducibility

The inhaled ^{99m}Tc -sestamibi clearance technique is a repeatable and safe technique with estimated radiation exposure of <0.5 mSv. Bland Altman analysis of the test and retest results confirmed excellent agreement between the values in 9 patients with a mean error of 4.8 minutes and precision of 5.4 minutes. In one patient a $>2\text{SD}$ deviation was observed. On further examination of records it was noted that the patient had stopped smoking between the first study when the inhaled ^{99m}Tc -sestamibi clearance was 155

minutes and the repeat study 3 weeks later when the clearance time had significantly reduced to 125 minutes. This is an interesting observation as it raises the possibility that smoking cessation may have led to the down regulation of MRP1 resulting in the faster inhaled sestamibi clearance which would suggest a possible causal relation between MRP1 expression and inhaled sestamibi clearance.

With the inclusion of the 10th patient the mean error was 7.1 minutes with a precision of 9.3 minutes. There was also excellent inter-observer reproducibility observed $p < 0.05$ supporting the robustness of the technique. (Table 2)

Interestingly a faster clearance of the inhaled sestamibi values were noted generally in the repeat study compared to the first study resulting in a positive bias as seen on the Bland –Altman analysis. The variance however is well within a single standard deviation in most cases. It is possible that some of this positive bias may be due to patient related factors as all other imaging factors remained constant. The patient factors may be due to better inhalation technique due to familiarity, reduced apprehension and possibly stopping smoking for longer period the second time around amongst others.

13. Limitations:

This study however is not without its limitations. The number of patients included in the study was small which makes interpretation of the results, although significant, at best a correlation rather than causal effect.

We carried out the ventilation of the patients in an upright position rather than the standard supine position used in routine lung scintigraphy. This may have an impact on the tracer distribution, particularly the upper zones due to the effect of gravity. The upright acquisition however ensured good compliance with the technique in some patients who found it hard to lie down and ventilate. However as the clearance was calculated over the whole lung, any effects of gravity did not affect the overall accuracy of the study.

We also did not look at possible regional variation of the clearance values and did not analyse the clearance specifically related to tumour tissue, given the increased MRP1 expression noted in lung tumours compared to the normal lung tissue. However it has to be noted that the histopathology samples were non-uniform and the size of pathology was too small, 1-4 cm only. This poses difficulty to correlate spatially with ROIs in the dynamic images which are also subject to patient motion, respiratory motion and thereby

affecting the spatial correlation. In addition, due to the influence of noise in the dynamic images (short time frames), the use of small ROIs would have lead to errors related to this analysis.

The best way of assessing the effect of different variables (Age, gender and smoking) on inhaled ^{99m}Tc -sestamibi clearance would have been to use multi factorial regression analysis. However, given the very strong influence of a single factor, smoking and the relatively small numbers of the patients in the other individual groups, using regression analysis was not possible which was a drawback in our study to assess the effect of various factors on inhaled ^{99m}Tc -sestamibi clearance. Future studies should be designed to assess this appropriately.

14. Future direction:

The results of the study raise many issues of which currently there is only limited understanding. Further larger studies correlating MRP1 expression with inhaled ^{99m}Tc -sestamibi clearance, smoking status and specific MRP1 blockers would help shed light on this complex issue possibly opening an exciting alternative dimension in the study of drug transporters in the lung. This may have the potential to play a significant role in optimising inhaled

drug delivery in various clinical scenarios including cancer and COPD.

15. Conclusion

The study results indicate a significant correlation between the predominant transporter in lung (broncho-alveolar epithelium) MRP1 and inhaled ^{99m}Tc -Sestamibi clearance. Inhaled ^{99m}Tc -Sestamibi clearance is a safe and reproducible technique and smoking is a significant factor affecting the lung clearance strongly, with age and gender as well playing significant roles. There is however no definite effect of environmental status on the clearance rate observed. Further studies are required to determine the potential applicability of these findings in clinical practice.

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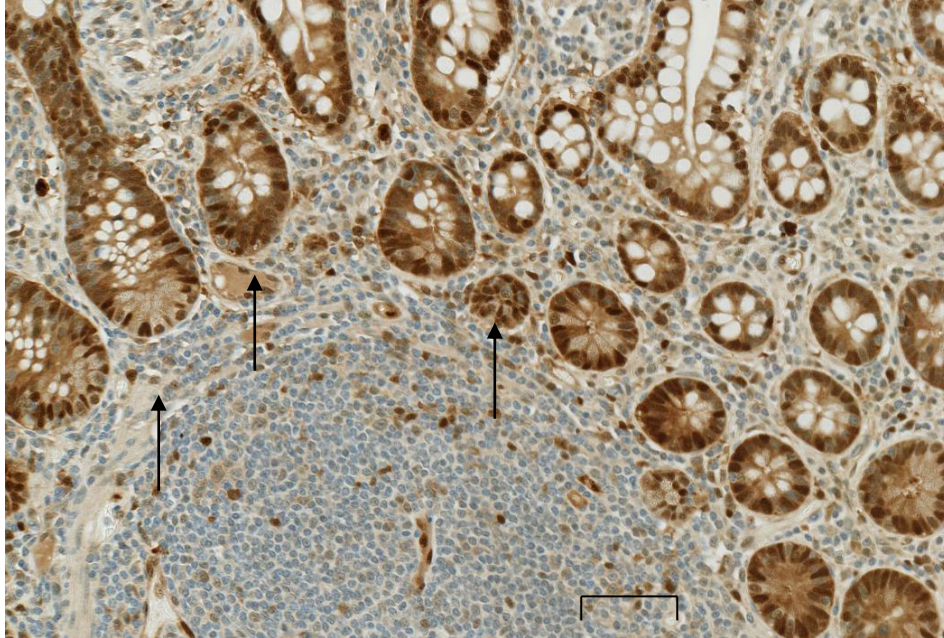
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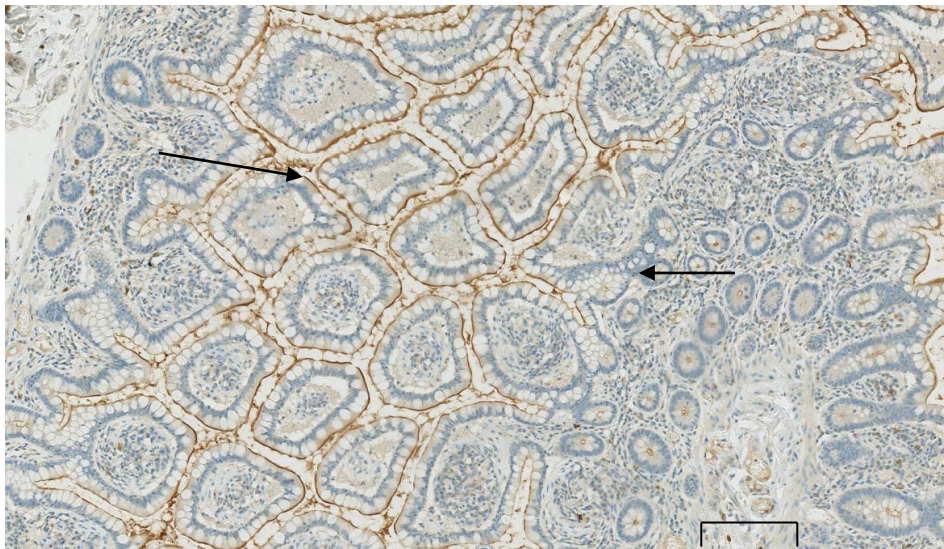
Figures

Fig 1a



Positive P-glycoprotein staining in the control Gut epithelium tissue (arrows pointing to intense brown staining within lining cells) Scale 200µm

Fig 1b



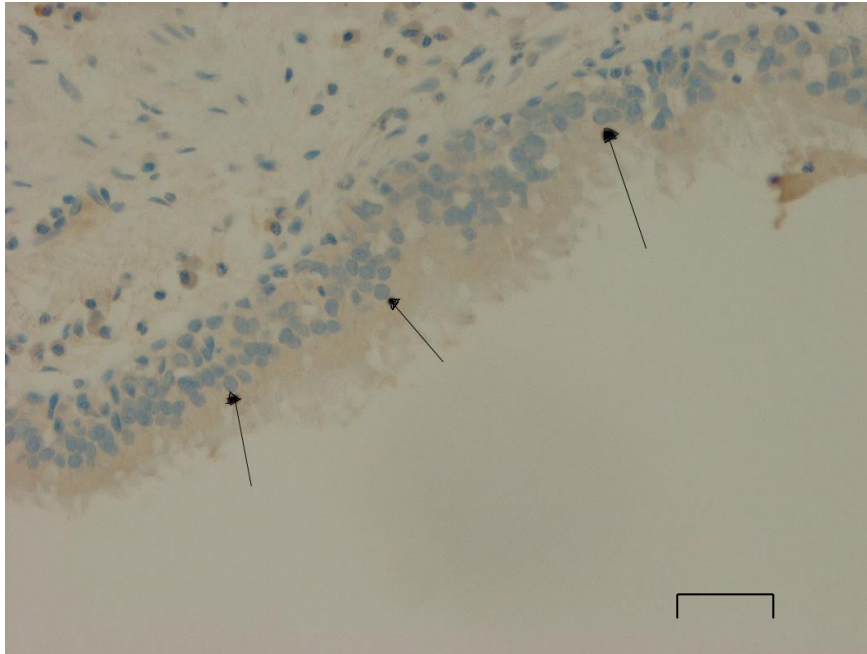
Positive Multidrug Resistance Protein 1 staining in the control Gut epithelium tissue (arrows pointing to intense brown staining within lining cells) Scale 200µm

Fig 1c



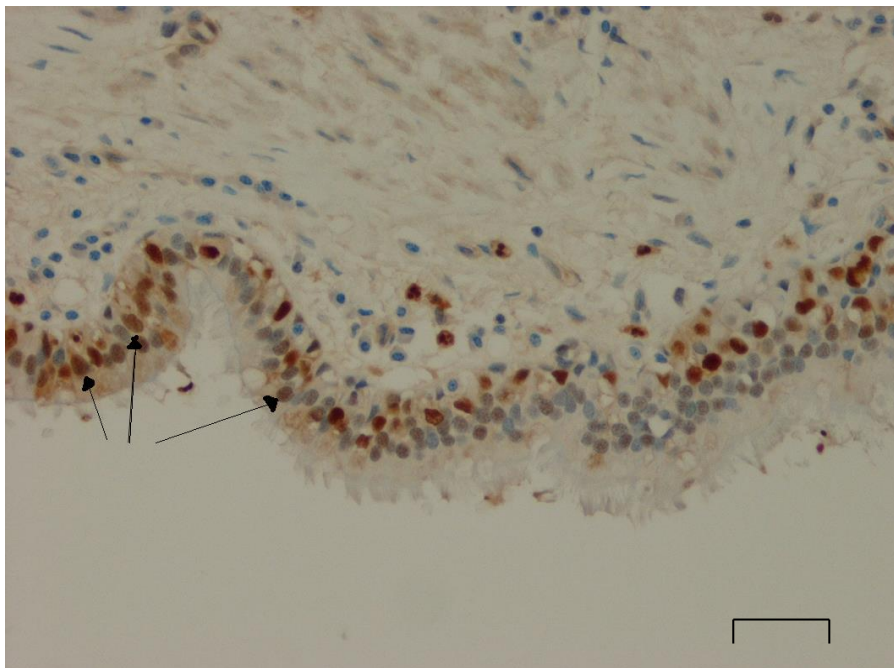
**Colour scale used to grade the strength of immunostaining.
0 – Negative 1 = Weak 2 = Moderate and 3 = Strong**

Fig 2a



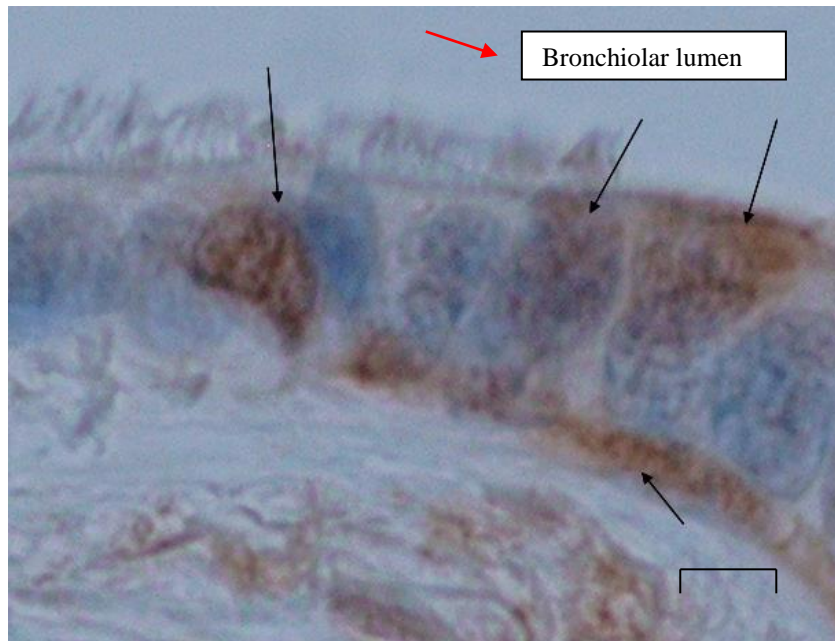
High power images of immunostaining of lung tissue in a patient clearly demonstrating negative P-gp staining with very little or no brown staining. (Arrows) Scale 25µm

Fig 2b



High power images of immunostaining of lung tissue in a patient clearly demonstrating intense brown staining for MRP1 (Arrows) Scale 25µm

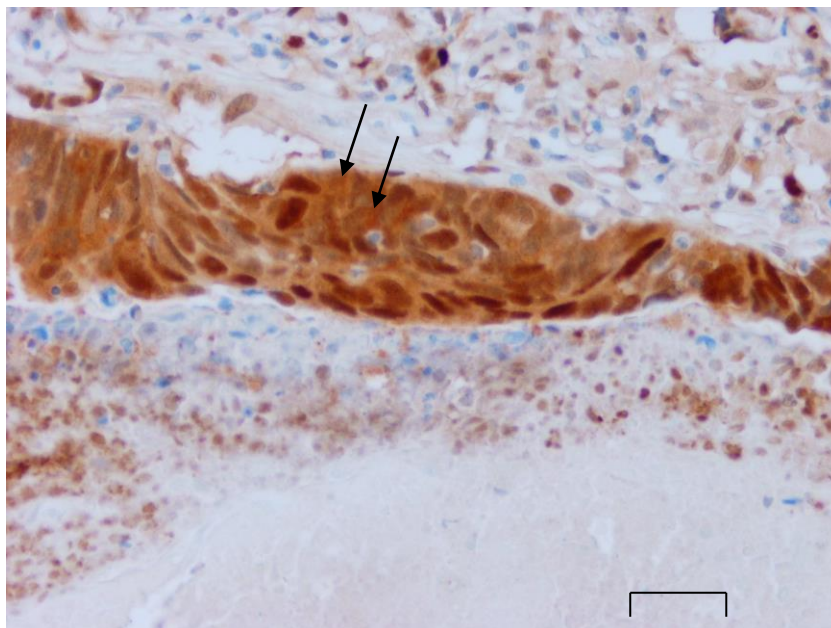
Fig 2c



High power images of immunostaining of lung tissue in a patient clearly demonstrating Grade 1 brown staining for MRP1 (Arrows)

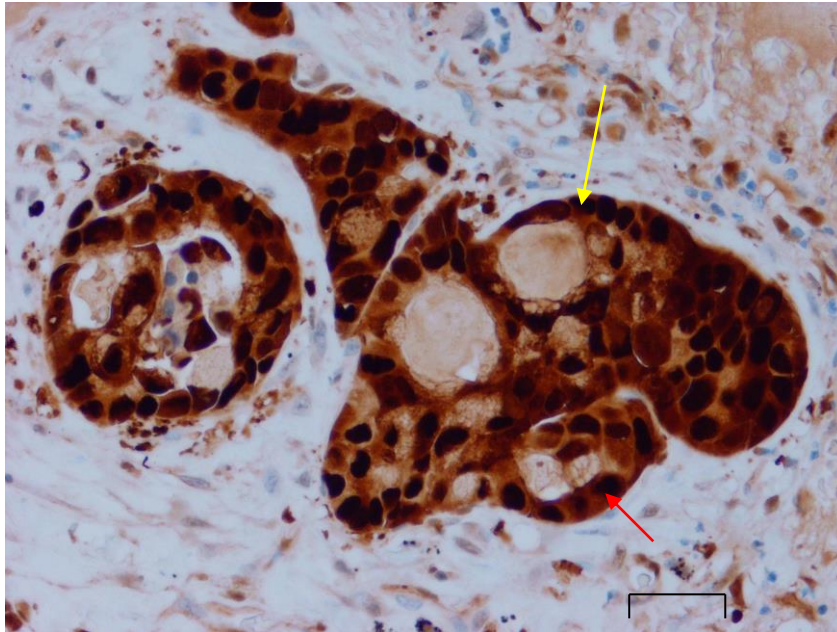
The black arrows also indicate the location of the MRP1 within the ciliated broncho-alveolar epithelium. The red arrow indicated the airway lumen space. Scale 400nm

Fig 2d



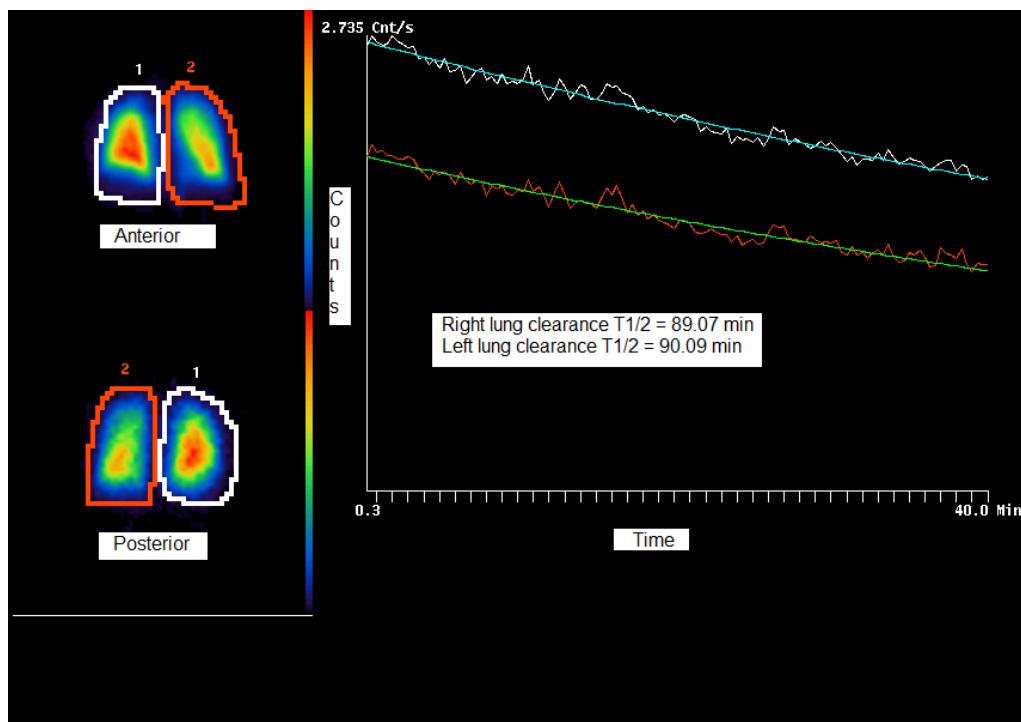
High power images of immunostaining of lung tissue in a patient clearly demonstrating Grade 2 brown staining for MRP1 (Arrows) Scale 10µm

Fig 2e



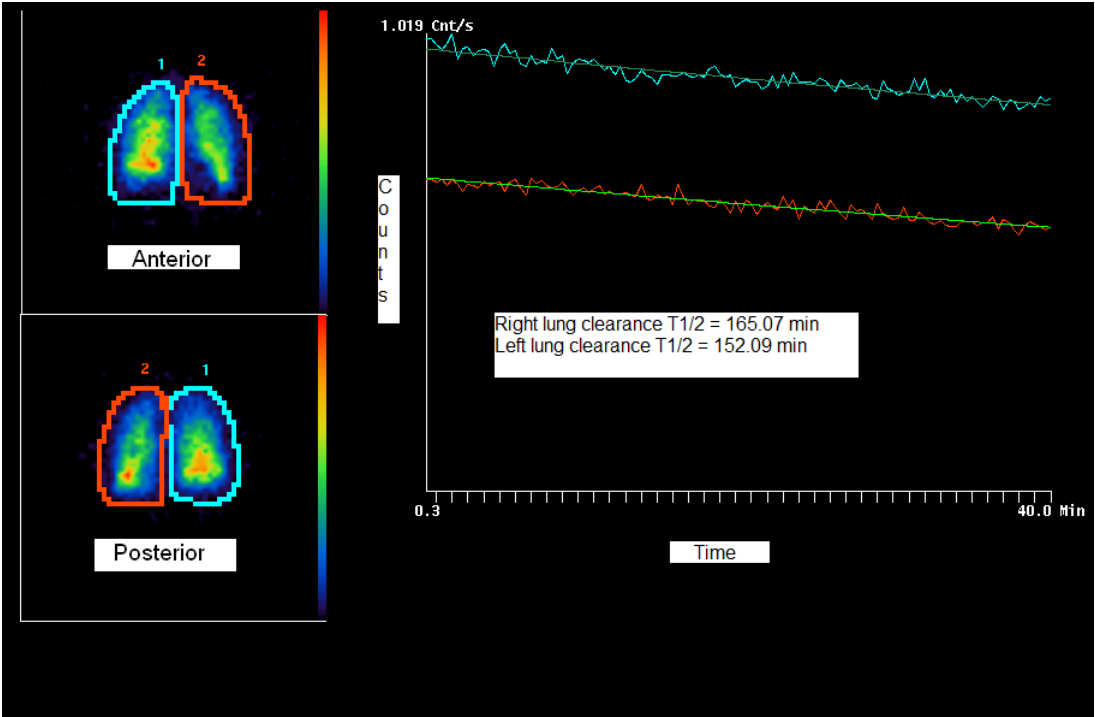
High power images of immunostaining of lung tissue in a patient clearly demonstrating intense Grade 3 brown staining for MRP1 (Arrows)
Scale 10 μ m

Fig 3a



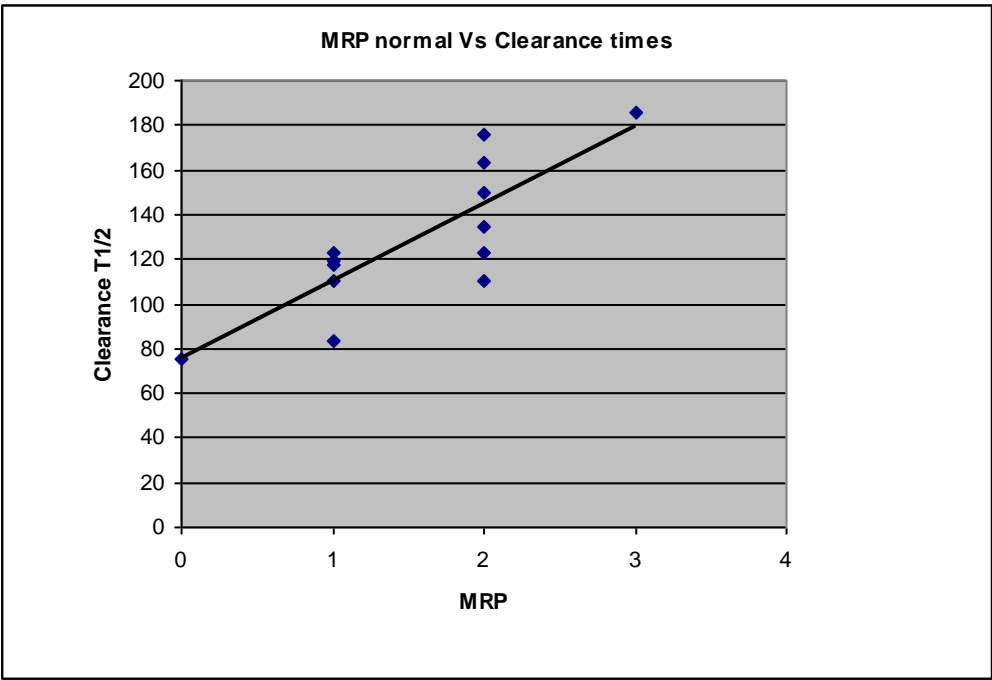
Inhaled ^{99m}Tc -sestamibi clearance in a patient with low MRP1 expression

Fig 3b



Prolonged inhaled ^{99m}Tc -sestamibi clearance in a patient with high MRP1 expression

Fig 3c



Correlation between grade of MRP1 expression and inhaled sestamibi clearance

Fig 4a

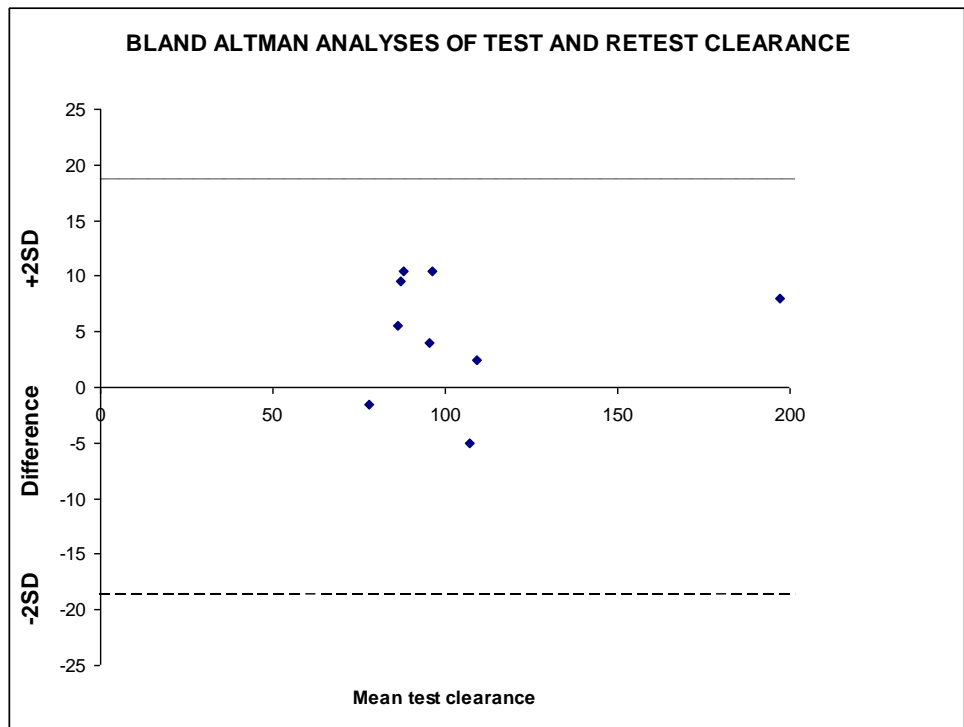
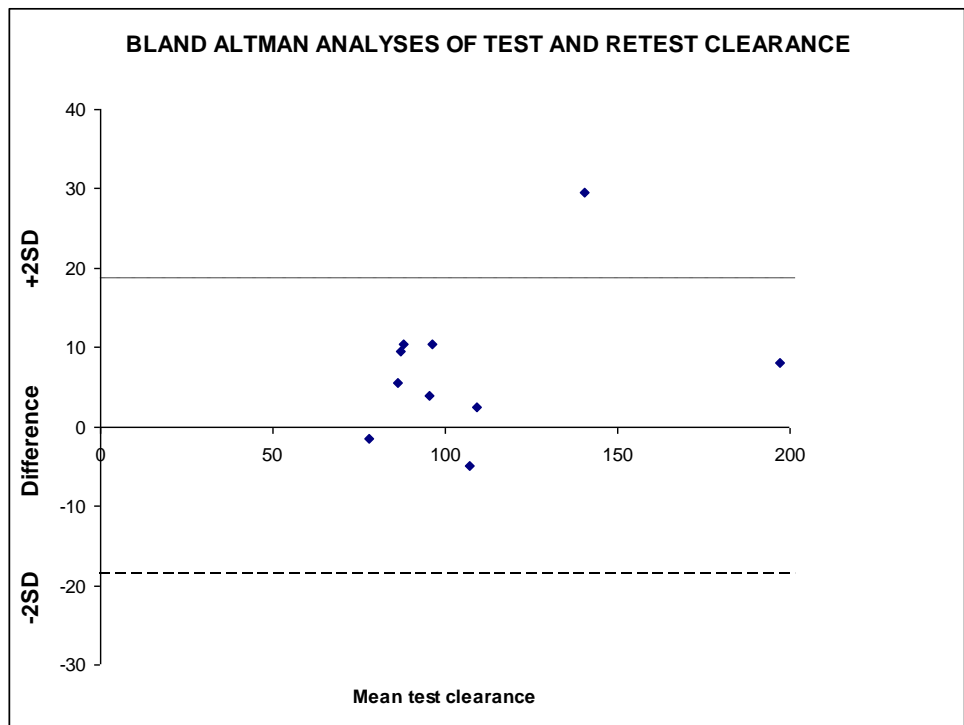
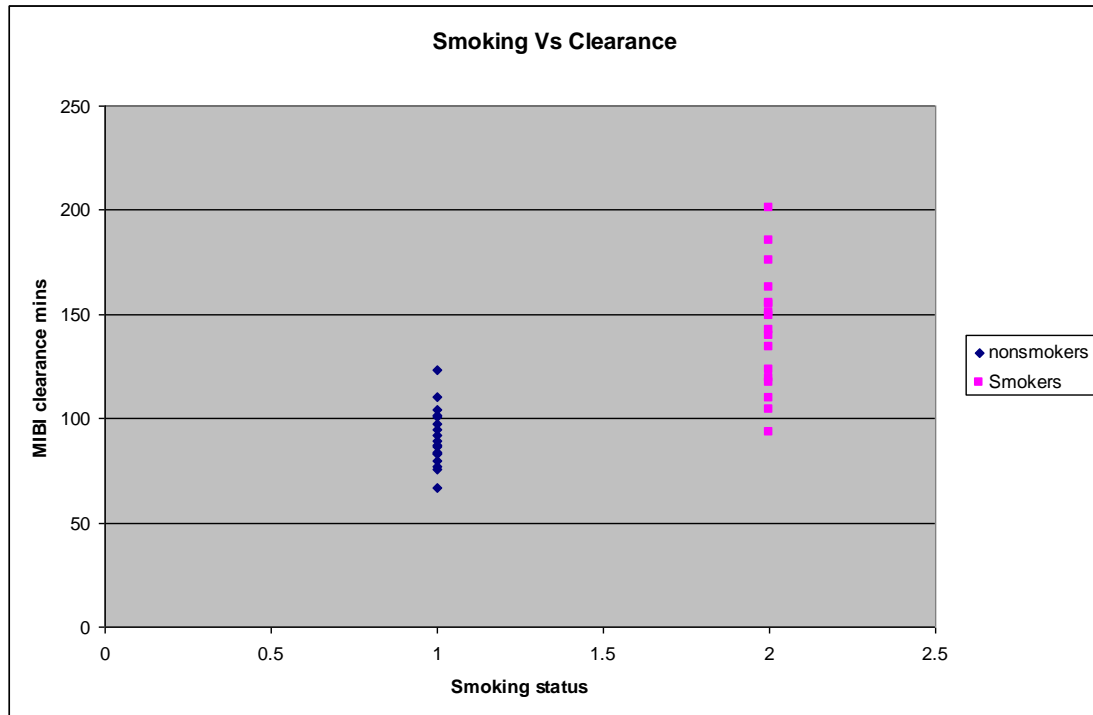


Fig 4b



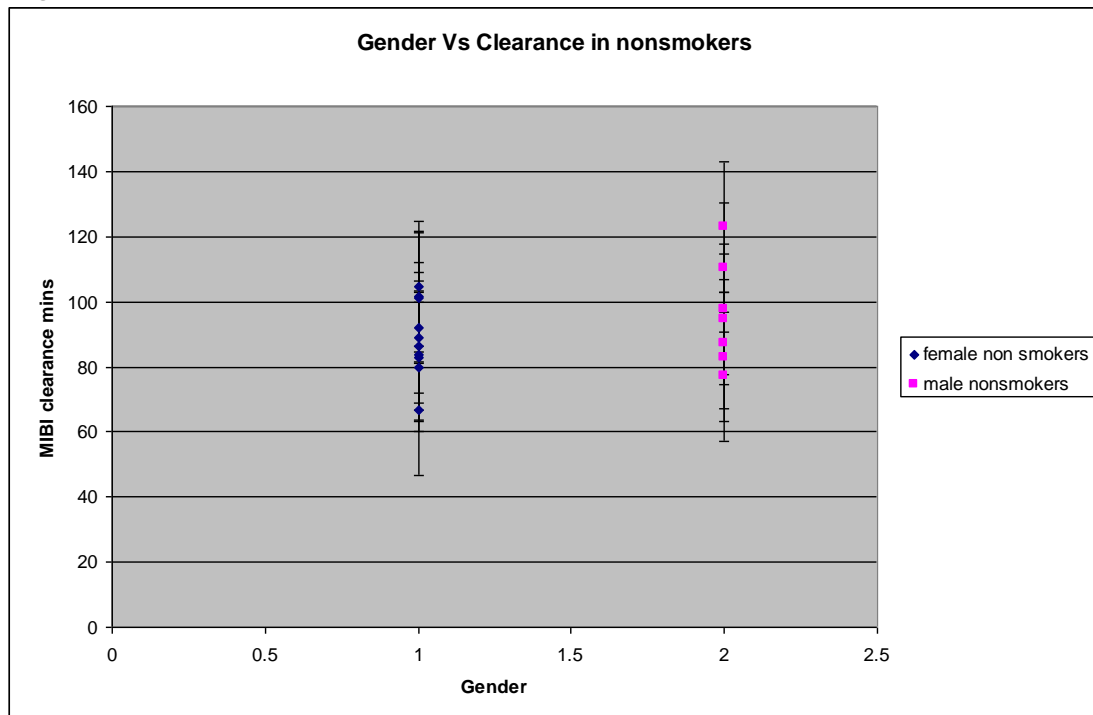
Bland-Altman analysis demonstrating excellent agreement between test and retest inhaled ^{99m}Tc -sestamibi clearance rates in 9 (4a) and 10 patients (4b).

Fig 5



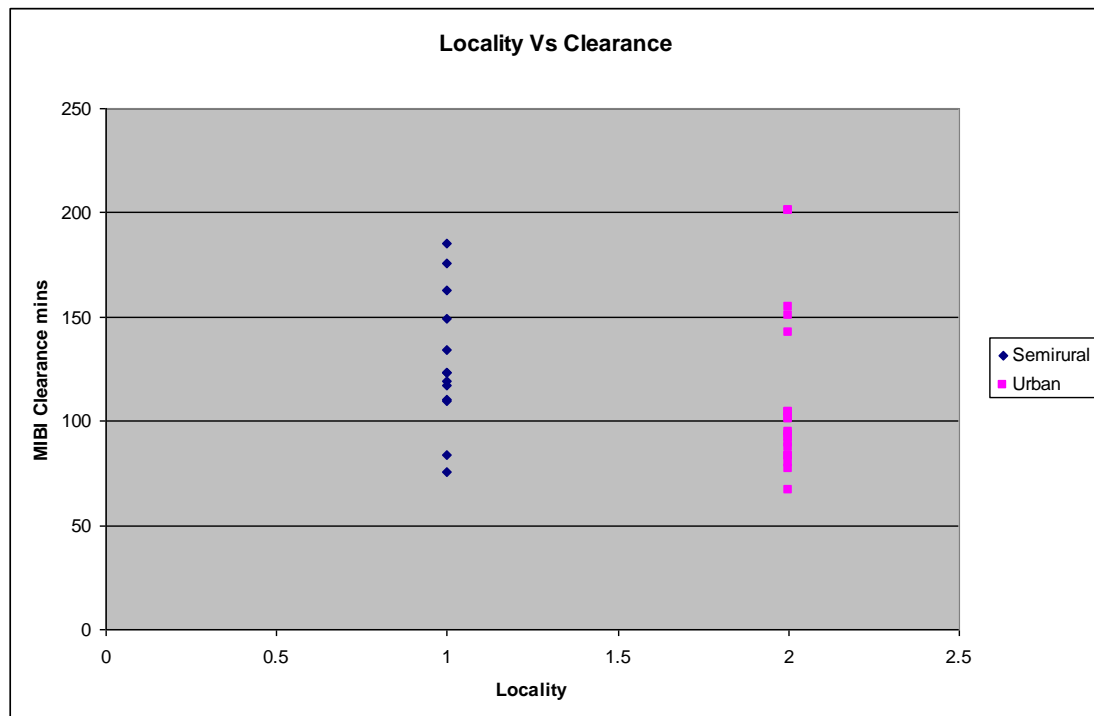
Smoking significantly prolonged clearance of inhaled sestamibi, Smokers, mean T1/2 = 142 +/- 29 minutes, compared to Non smokers, mean T1/2 = 91 +/- 14 minutes. $p < 0.0001$. (Fig 5)

Fig 6



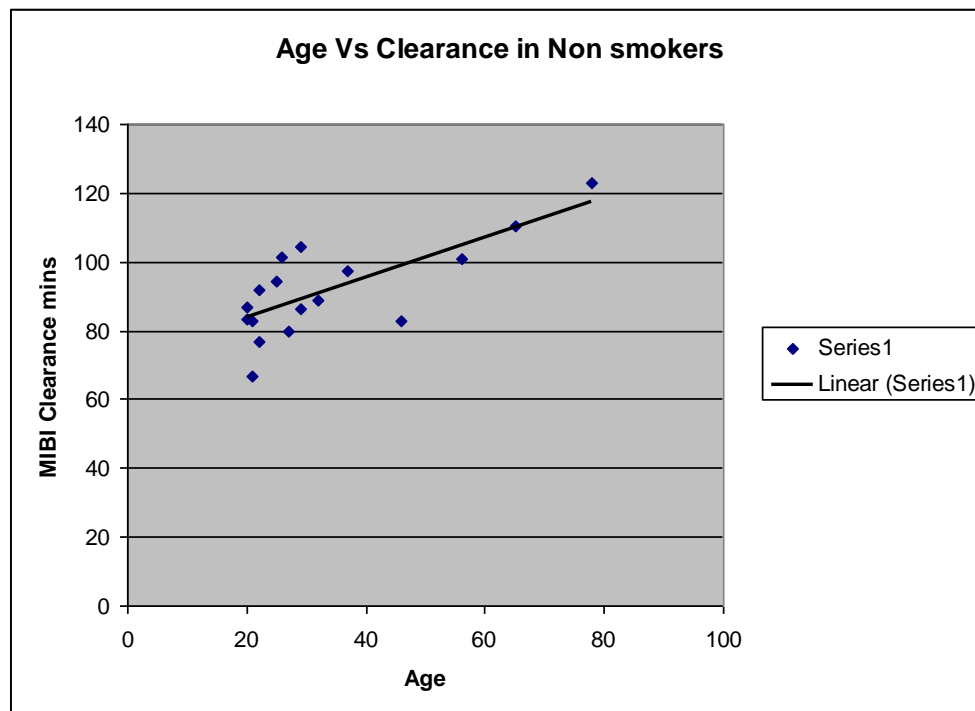
Male participants demonstrated significant delayed clearance ($T_{1/2} = 128 \pm 36$ minutes) when compared to females mean $T_{1/2} = 105 \pm 28$ minutes. $p < 0.05$. (Fig 6)

Fig 7



There was however no significant difference noted between those living London (urban), mean T1/2 = 89 +/- 11 minutes and those in Semi rural area, mean T1/2 = 96 +/- 20 minutes. $p>0.05$. (Fig 7)

Fig 8



Increasing age resulted in delayed inhaled sestamibi clearance; >30yrs age, mean T1/2 = 134 +/- 31 minutes compared to <30 yrs, mean T1/2 = 106 +/-34 minutes. $p < 0.05$. (Fig 8)

Fig 9

Arrows depicting tracer in Kidneys

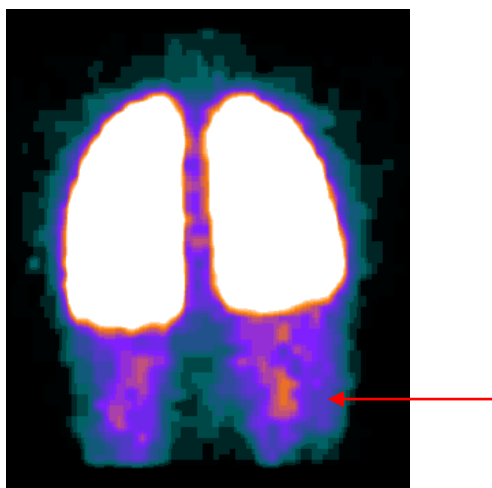


Figure demonstrating the presence of the tracer in the systemic circulation

Table 1 – Inhaled MIBI clearance for all patients

Rt lung clearance	Lt lung clearance	Mean
78	88	83
102	101	101.5
82	84	83
99	103	101
95	89	92
78	89	83.5
80	80	80
85	88	86.5
98	91	94.5
86	88	87
88	90	89
107	102	104.5
77	77	77
67	66	66.5
105	116	110.5
75	76	75.5
105	90	97.5
145	101	123
91	95	93
147	138	142.5
151	150	150.5
203	199	201
104	105	104.5
138	141	139.5
150	160	155
158	151	154.5
201	170	185.5
123	115	119
157	142	149.5
129	140	134.5
127	119	123
113	107	110
120	115	117.5
173	178	175.5
188	138	163

Values of individual inhaled ^{99m}Tc -sestamibi clearance for each lung and the mean lung clearance for each patient. A Spearman Rho calculation confirmed an excellent correlation between the individual clearances between right and left lung with an R value 0.95 and $p < 0.05$.

Table 2a – Inhaled MIBI clearance Reviewer 1 Vs Reviewer 2

Physician 1 Clearance measurement Individual lungs	Physician 2 Clearance measurement Individual lungs
76	77
108	105
84.5	91
211	203
95	102
73	77
129	116
92	95
214	199
99	101

Table 2b

Physician 1 Mean of both lung Clearance measurements	Physician 2 Mean of both lung Clearance measurements
74.5	77
118.5	110.5
88.25	93
212.5	201
97	101.5

Values of lung clearances as calculated by 2 experienced nuclear medicine physicians. A Spearman Rho calculation confirmed an excellent correlation between the individual clearances with an R value 0.97 and $p < 0.05$. The lung clearance values measured as a mean value (both lungs) also demonstrate excellent correlation between the 2 reviewers, R value 0.99 and $p < 0.001$